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ORIGINAL PAPER

Methyl- and acetyltransferases are stable epigenetic markers postmortem

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Abstract Postmortem brain tissue has been reported to be suitable to delineate regional pattern of possible disturbances underlying epigenetic functionality. However, from many parameters that have been detected in postmortem brain regions it is noteworthy that an effect of postmortem interval (PMI), storage time and premortem parameters should not be underestimated. Our previous investigation revealed that tryptophan (TRP) levels in postmortem brain tissue is affected by PMI and storage time. Since, alteration in TRP levels are assumed to be due to protein degradation, we further investigated whether TRP correlates to variables such as RNA, proteins and DNA modulators. In addition, we aimed to elucidate whether established postmortem variables may influence epigenetic parameters. These were investigated in well characterized postmortem human brain tissue originating from the European Brain Bank consortium II (BNEII). We could confirm previous findings, in which some protein levels alter because of prolonged PMI. Similarly, we demonstrated an influence of increased storage period on TRP levels, which might indicate degradation of proteins. Still not all proteins degrade in a similar manner, therefore a specific analysis for the protein of interest would be

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recommended. We found that methyltransferase- and acetyltransferase-activities were relatively preserved with PMI and storage duration. In conclusion, preservation of acetyltransferase- and methyltransferase-activities provides possible evidence of stability for epigenetic studies using postmortem tissue.

Keywords Acetyltransferase · GFAP · Methyltransferase · Postmortem · RNA · TAU · Tryptophan · α-Tubulin

Introduction

Postmortem brain tissue has been reported to be suitable to delineate regional pattern of possible disturbances underlying epigenetic functionality (Keller et al. 2010; Barrachina and Ferrer 2009; Zhubi et al. 2009; McGowan et al. 2009; Ernst et al. 2009; Tamura et al. 2007). However, several studies report an effect of postmortem interval (PMI), storage time and premortem variables on human postmortem brain tissue preservation of DNA, RNA and proteins (Ferrer et al. 2008; Beach et al. 2008; Waldvogel et al. 2008; Grinberg et al. 2007; Chandana et al. 2009). To reduce any false positive or negative results it is important to check the biomarker of interest that best mimics the brain region (Kretzschmar 2009). The use of advanced molecular and biochemical methods for studying well preserved and well characterized human brain tissue can provide new knowledge regarding the cascade of events causing neurological or psychiatric disorders. Therefore, Brain Banks are, without any doubt, most valuable for researchers in this field. In the past, there were several limitations and debate concerning brain-banking protocols, especially regarding tissue quality control (Kretzschmar 2009; Bell et al. 2008; Schmitt et al. 2007; Pope et al. 1997).

Premortem and postmortem parameters, such as agonal state or PMI were studied more recently for their influence on DNA, RNA, protein levels, and enzyme quality (Grünblatt et al. 2009; Monoranu et al. 2009; Ferrer et al. 2007a, b; Ervin et al. 2007; Buesa et al. 2004). Preservation of DNA methylation in several gene promoters has recently also been investigated in postmortem brain (Barrachina and Ferrer 2009). In our recent study, we could show that tryptophan (TRP) levels in brain tissue correlate to PMI and storage duration. Since we postulated that elevated TRP may mirror increased protein degradation as consequence of prolonged PMI and storage duration the question arose whether high and low TRP levels correlate to certain parameters such as RNA, protein and epigenetic markers. Therefore, selected samples with high and low levels of TRP in well characterized postmortem human brain tissue originating from the European Brain Bank consortium II (BNEII) were studied. Correlations have been examined between PMI, storage duration and RNA quality; neurochemical markers such as TRP concentration, glutamate decarboxylase (GAD; EC 4.1.1.15) and phosphofructokinase-1 (PFK; EC 2.7.1.11) enzyme activities (Grünblatt et al. 2009); tissue pH (Monoranu et al. 2009); protein levels/degradation of selected proteins involved in neurodegenerative diseases and psychiatric disorders; and enzymatic activities of acetyltransferase and methyltransferase which are involved in the process of DNA acetylation and methylation (epigenetic markers). Through these association studies, we aim to elucidate whether TRP levels do correlate to protein degradation as well as to RNA and DNA-modulator stability. In addition since the stability of epigenetic markers in postmortem brain tissue is not yet known, we explored this possibility.

Materials and methods

Brain tissue samples

Postmortem human brain tissue was obtained from 3 European Brain Bank Centers (BBCs) organized

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in BNE II: London, Würzburg Department of Neuropathology (NP) and Göttingen (Bell et al. 2008). Whole human brains were obtained with the consent of relatives according to the guidelines of the NIH Guide for Use of human tissue (NIH 2001) and were approved by the national and local ethics committees. Cause of death was determined from clinical notes, the autopsy report, or the death certificate.

In preparation for routine neuropathological examination, the brain was divided midsagittally, and one half-brain was immersed in 4.5% paraformaldehyde (Fischar GmbH, Saarbrücken, Germany) for 3–4 weeks. The remaining half was cryopreserved following coronal slicing for cortical sampling, sagittal slicing for sampling the cerebellum and transverse sectioning of the brain stem. Tissue samples were snap frozen on brass plates, cooled on dry ice, and stored at −80°C until requested for experimental use. A neuropathological assessment was completed by macroscopic and histological examination of paraffin-embedded tissue. Because the tissue was obtained from different institutions, detailed information concerning tissue preparation and storage was collected. All samples described in this study were shipped on dry ice in frozen state without any defrost phase, and were processed and analyzed in the Neurochemistry Laboratory of the Clinic for Psychiatry, University Würzburg (Germany) as well as in the Institute of Neuropathology, University Barcelona (Spain).

Twelve cases were available (Table 1), with 'control' defined as no history of any neurological or neuropsychiatric disease plus confirmation by histopathological examination. The gender distribution was balanced with samples from 6 males and 6 females. After determining that brain region had no effect on TRP levels or on GAD or PFK activity, the mean values for each case were calculated using measurements from the different brain regions (Grünblatt et al. 2009). Detailed information about age (ranging from 32 to 84 years), PMI (range from 5 to 100 h) and storage time (range from 9 to 120 months) was provided for all cases. Sample characteristics are summarized in Table 1.

pH measurements

Tissue pH values were determined for fresh dissected tissue or for thawed frozen blocks of tissue using a

Table 1 Demographic characteristics for all cases

No.	Case	Sex	Storage (months)	PMI (hours)	Age (years)	Agonal state	Dead body storage temperature (°C)	Freezing method	Tissue packaging method	Clinical/pathological diagnosis	BBC
1	S037/05	M	9	14.0	49	Prolonged	4	Dry ice	Aluminum foil	Ruptured spleen (control)	Würzburg NP
2	S111/03	M	27	5.0	67	Rapid	4	Dry ice	Aluminum foil	Myocardial infarction (control)	Würzburg NP
3	S133/03	M	26	48.0	84	Rapid	4	Dry ice	Aluminum foil	Myocardial infarction (control)	Würzburg NP
4	S242/00	M	58	12.0	32	Rapid	4	Dry ice	Aluminum foil	Myocarditis (control)	Würzburg NP
5	A090/97	F	108	48.0	54	Rapid	4	Dry ice	Plastic bags	Lung embolism (control)	London
6	A224/96	F	120	60.0	64	Prolonged	4	Dry ice	Plastic bags	Bladder carcinoma (control)	London
7	A284/98	M	96	26.0	57	Rapid	4	Dry ice	Plastic bags	Global ischemia, ruptured aortic aneurysm	London
8	A003/98	F	96	100.0	69	Rapid	4	Dry ice	Plastic bags	Global ischemia, myocardial infarct ion	London
9	A309/99	F	84	21.0	58	Prolonged	4	Dry ice	Plastic bags	Global ischemia, bronchial carcinoma	London
10	51/02	M	60	24.0	57	Rapid	4	Isopentane	Plastic bags	Myocardial infarction (control)	Göttingen
11	61/01	F	73	16.0	66	Rapid	4	Isopentane	Plastic bags	Myocardial infarction (control)	Göttingen
12	39/03	F	48	32	77	Rapid	4	Isopentane	Plastic bags	Lung embolism, Schizophrenia	Göttingen

NA not available, *F* female, *M* male, *NP* neuropathology, *BBC* brain bank center, *PMI* postmortem interval

pH meter as previously described (Grünblatt et al. 2009; Monoranu et al. 2009).

TRP concentration determination

The TRP measurements were conducted according to the method described previously for tissue-free amino acid determination (Grünblatt et al. 2009). TRP tissue levels were calculated for all samples in μM concentration (all samples were of 52.63 mg tissue/ml).

GAD activity assay

GAD activity was determined by measuring the production of radiolabeled carbon dioxide from ^{14}C-glutamate as described previously (Grünblatt et al. 2009). Assays were conducted in triplicate for each sample, and results were normalized for the total protein content measured via Bradford (Bradford 1976). Enzyme activity was expressed as nmol/mg protein/hour.

PFK activity assay

Determination of PFK activity by measuring the production of radiolabeled D-fructose-1,6-biphosphate from [γ-^{32}P]-ATP was performed as described previously (Grünblatt et al. 2009). Assays were conducted in triplicate for each sample, and results were normalized for total protein content measured via Bradford. Enzyme activity was expressed as nmol fructose-1,6-bisphosphate/mg protein/minute.

Western blot analysis

Gel electrophoresis and western blotting was carried out as reported elsewhere (Ferrer et al. 2007b). The following proteins were analyzed with appropriate antibodies: RAB3A (rabbit polyclonal, Santa Cruz Biotechnology, Heidelberg, Germany, diluted 1:200), synaptophysin (mouse monoclonal, Dako, Dakopats, Barcelona, Spain diluted 1:6,000),α-synuclein (mouse monoclonal, Neomarkers, Fredmont, CA, USA, diluted 1:3,000),α-synuclein (rabbit polyclonal, Chemicon, Barcelona, Spain, diluted 1:2,000), glial fibrillary acidic protein (GFAP, mouse monoclonal, Dako, diluted 1:30,000), TAU-P-ser262 (rabbit polyclonal, Calbiochem, LaJolla, CA, USA, diluted 1:500), TAU46 (mouse monoclonal, Abcam, Cambridge, UK, diluted 1:1:1,000), calcium calmodulin kinase IIα(CamK-IIα, rabbit polyclonal, Santa Cruz, diluted 1:500) α-Tubulin (mouse monoclonal, Sigma, Madrid, Spain, dilution 1:10,000), β-Tubulin (rabbit polyclonal, Abcam, diluted 1:10,000), metabotropic glutamate receptor 2–3 (mGlu R2/R3, rabbit polyclonal, Upstate, Millipore, Barcelona, Spain, diluted 1:1,000), TrkB (rabbit polyclonal, Santa Cruz, diluted 1:200) and brain derived neurotrophic factor (BDNF, rabbit polyclonal Santa Cruz, diluted 1: 1,000) normalized all with β-actin (rabbit polyclonal, Dako, diluted 1: 20,000). Protein expression levels were determined by densitometry of the bands using Total Laboratory v2.01 software. This software detects the bands obtained in Western blots and gives individual values which are dependent on the light quantification of the corresponding band. Measurements are expressed as arbitrary units. The results were normalized for β-actin densitometry.

RNA quality analysis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA purity was assessed using the A260/A280 ratio (A260 = absorbance at 260 nm; A280 = absorbance at 280 nm). RNA integrity was analyzed on an Agilent 2100 Bioanalyzer, which also automatically calculates the sample concentration, the 28S/18S ribosomal ratio, and the RNA Integrity Number (RIN). RIN values lower than 7 are usually regarded as degraded RNA/low RNA quality, while values over 7 are regarded as good RNA quality.

Acetyltransferase activity

Total acetyltransferase activity was measured according to the Acetyltransferase Activity Kit (Assay Designs Inc. Michigan, USA). For homogenate preparation small frozen brain tissue were added in 1× RIPA buffer in a 1: 10 (weight to volume) dilution and lysed using Tissue Lyser (Qiagen, Hilden, Germany) at 25 Hz for 30 min. Total protein was determined via Bradford. The acetyltransferase activity test was run in triplicates with total protein concentration of 1 ng/μl. An end-point measurement was performed and fluorescence was read at 380ex/520em according to the manufacture's instructions.

Methyltransferase activity

Total methyltransferase activity was measured according to the Methyltransferase Activity Kit (Assay Designs Inc. Michigan, USA). Homogenate preparation and measurements were performed as the acetyltransferase activity assay.

Statistical methods

For analysis of high and low TRP levels on various parameters one-way ANOVA was used. Correlations were calculated using Spearman's correlation coefficient. *P*-values lower than 0.05 were considered statistically significant. The analysis was conducted using SPSS 15.0 software (SPSS Inc. Headquarters, Chicago, IL, USA).

Results

TRP correlation to postmortem factors

In this study we selected six brain tissue samples with high levels of TRP (213 ± 30.7 μM) and six with low TRP levels (31.3 ± 16.6; $P < 0.0001$; Supplementary Table S1). Gender, agonal state (rapid versus prolonged) and freezing method did not affect TRP levels (Supplementary Table S1). We could observe significantly longer storage duration for the group of high TRP levels in comparison to the low TRP (94 ± 20.7 months; 40.2 ± 23.7 months; respectively, $P = 0.002$; Supplementary Table S2). Nominal significant longer PMI was observed for the high TRP group versus the low TRP group (46.5 ± 30.4; 21.2 ± 15.9 h; respectively, $P = 0.101$; Supplementary Table S2). Age, pH value, PFK, GAD activity, RNA quality and protein variables did not affect TRP levels when divided into high- and low-levels (Supplementary Table S2; for raw data see supplementary Table S3).

Effects of PMI and storage duration on preservation of RNA, neurochemical and protein markers

Spearman correlation analysis was conducted between postmortem parameters such as storage period and PMI to neurochemical parameters commonly measured by scientists in brain tissue. Table 2 summarizes the correlation results between selected markers (for all correlations see supplementary Table S4). TRP levels significantly correlated positive to storage period (Rho = 0.697, $P = 0.008$). TAU46, TAU-p ser262 and α-Tubulin protein levels measured via western blotting significantly correlated with PMI (Rho = −0.669, −0.634, −0.636; $P = 0.017$, 0.027, 0.035, respectively). All three proteins show decrease in their levels with longer periods of PMI.

Brain tissue pH-values did not show any correlation to any of the markers studied. RNA concentration and RNA quality measured as RIN were found to correlate with each other (Rho = 0.558, $P = 0.047$), which means RNA quality increases with RNA concentration in postmortem brain tissue. But RNA quality did not alter with prolonged PMI or storage duration (Supplementary Table S4).

Effects of PMI, storage duration and enzyme activity on preservation of epigenetic markers

In order to study whether methyl- and acetyltransferase activities, as epigenetic markers, alter as a result of postmortem factors, we correlated enzyme activity at different PMI and to storage duration. No modifications in acetyltransferase activity and methyltransferase activity were noticed with increasing postmortem delay or duration of storage (Table 2).

We found a correlation between GAD activity to the methyltransferase activity (Rho = 0.698, $P = 0.008$), in which increase of one follows the other (Table 2).

Correlations between protein preservation

Regarding protein levels in postmortem brain tissue, we found correlations between several proteins. Synaptophysin levels increase with increased mGlu R2/R3 levels (Rho = 0.773, $P = 0.005$). Increased GFAP levels correlated significantly with decreased levels of α-Synuclein, TAU46 and α-Tubulin (Rho = −0.836, −0.636, −0.827, $P = 0.001$, 0.035, 0.002, respectively). In addition, TAU46 levels correlate significantly with α-Synuclein, TAU-P ser262 and α-Tubulin (Rho = 0.827, 0.647, 0.827, $P = 0.002$, 0.02, 0.002, respectively), in which increased levels of TAU46 associate with increased levels of the listed proteins. Similarly to TAU46, α-Tubulin was significantly correlated with α-Synuclein and TAU-P ser262 (Rho = 0.855, 0.700, $P = 0.001$, 0.016, respectively).

Table 2 Spearman correlation coefficients between affected parameters

		Storage	PMI	TRP	GFAP	TAU46	TAU-P-ser262	α-Tubulin	Acetyltransferase activity	Methyltransferase activity
Storage	Rho	1	0.552	**0.697****	0.082	−0.137	−0.575	−0.26	0.532	−0.209
	P-value	–	0.05	**0.008**	0.811	0.672	0.05	0.441	0.061	0.492
	N	12	12	**12**	11	12	12	11	12	12
PMI	Rho	0.552	1	0.438	0.364	**−0.669***	**−0.634***	**−0.636***	0.124	−0.281
	P-value	0.05	–	0.134	0.272	**0.017**	**0.027**	**0.035**	0.687	0.352
	N	12	12	12	11	**12**	**12**	**11**	12	12
TRP	Rho	**0.697****	0.438	1	−0.209	0.126	−0.301	−0.127	0.412	−0.022
	P-value	**0.008**	0.134	–	0.537	0.697	0.342	0.709	0.162	0.943
	N	**12**	12	12	11	12	12	11	12	12
GFAP	Rho	0.082	0.364	−0.209	1	**−0.636***	−0.318	**−0.827****	−0.055	−0.1
	P-value	0.811	0.272	0.537	–	**0.035**	0.34	**0.002**	0.873	0.77
	N	11	11	11	11	**11**	11	**11**	11	11
TAU46	Rho	−0.137	**−0.669***	0.126	**−0.636***	1	**0.657***	**.827****	0.189	0.133
	P-value	0.672	**0.017**	0.697	**0.035**	–	**0.02**	**0.002**	0.557	0.681
	N	12	**12**	12	**11**	12	**12**	**11**	12	12
TAU-P-ser262	Rho	−0.575	**−0.634***	−0.301	−0.318	**0.657***	1	**0.700***	−0.112	0.133
	P-value	0.05	**0.027**	0.342	0.34	**0.02**	–	**0.016**	0.729	0.681
	N	12	**12**	12	11	**12**	12	**11**	12	12
α-Tubulin	Rho	−0.26	**−0.636***	−0.127	**−0.827****	**0.827****	**0.700***	1	−0.064	0.109
	P-value	0.441	**0.035**	0.709	**0.002**	**0.002**	**0.016**	–	0.853	0.75
	N	11	**11**	11	**11**	**11**	**11**	11	11	11
Acetyltransferase activity	Rho	0.532	0.124	0.412	−0.055	0.189	−0.112	−0.064	1	−0.044
	P-value	0.061	0.687	0.162	0.873	0.557	0.729	0.853	–	0.887
	N	12	12	12	11	12	12	11	12	12
Methyltransferase activity	Rho	−0.209	−0.281	−0.022	−0.1	0.133	0.133	0.109	−0.044	1
	P-value	0.492	0.352	0.943	0.77	0.681	0.681	0.75	0.887	–
	N	12	12	12	11	12	12	11	12	12

Significant results are marked as bold

** *P*-value < 0.01; * *P*-value < 0.05. Storage was measured in months, Postmortem interval (PMI) in hours, tryptophan (TRP) in μM, glial fibrillary acidic protein (GFAP), TAU46, TAU-P ser262, α-Tubulin, in density normalized to *β*-actin, Acetyltransferase activity and Methyltransferase activity in RFU. For detailed information see supplementary Table S3 and S4

TrkB levels correlated to increased levels of β-Tubulin and mGlu R2/R3 (Rho = 0.819, 0.709, $P = 0.001$, 0.007, respectively). As well as, β-Tubulin correlated significantly to mGlu R2/R3 (Rho = 0.797, $P = 0.001$; Table 2; Supplementary Table S4).

Discussion

In the current study, we demonstrated again that TRP levels may be used as a marker for PMI and storage duration of postmortem brain tissue quality. Increased TRP levels in postmortem tissue seem to indicate protein degradation and enzyme activity alterations, as previously reported (Ferrer et al. 2007a; Birkmayer and Riederer 1980). This finding was concomitant with degradation of some proteins, TAU-P-ser262 and α-tubulin that were significantly reduced as a result of increased PMI. This finding is confirmation of previously published studies demonstrating degradation of proteins with prolonged PMI (Ferrer et al. 2007a; Crecelius et al. 2008; Santpere et al. 2006). However, it is notable that not all proteins degrade in a similar manner (Omalu et al. 2005); therefore scientists should test the protein/enzyme of interest ahead for its sensitivity to degrade as consequence of pre-mortem of postmortem conditions.

The importance of RNA quality in human postmortem brains research is not questionable (Ferrer et al. 2008; Ervin et al. 2007; Barton et al. 1993; Hynd et al. 2003; Tomita et al. 2004; Barrachina et al. 2006; Chevyreva et al. 2008; Popova et al. 2008). In our study, we could not find any direct influence of postmortem variables (e.g. PMI, storage, pH-value, or neurochemical parameters) on the RNA integrity measured by RIN values. This finding is in accordance to several reports indicating no correlation to these parameters (Ferrer et al. 2008; Ervin et al. 2007; Popova et al. 2008; Stan et al. 2006). Still, the range of PMI in our study was not lower than 5 h which might bias our results since RNA degradation occurs rapidly, while others did not confirm this (Perrett et al. 1988).

Regarding correlations among proteins, the present results serve to point out that special care must be taken when considering protein–protein interactions and construction of protein networks in specific conditions, based on data obtained from postmortem human brain material. It is clear that the reconstruction of protein networks and metabolic pathways is a useful tool to increase understanding about altered or preserved metabolic pathways in normal and pathological conditions (Xue et al. 2007; Mohajerani et al. 2007). Yet, the construction of maps and networks and more particularly the meaning of decreased or increased protein levels can be largely hampered by postmortem artifacts due to selective individual vulnerability of proteins to premortem and postmortem parameters. The study of protein conservation with PMI was to investigate whether there is a common pattern of degradation among similar groups of proteins or proteins related to a particular pathway. The present findings show correlations in the expression levels among certain cytoskeletal proteins, synaptic and receptor proteins (especially TAU46, TAU-p ser262 and α-Tubulin were degraded with increased PMI) and we hypothesize that other proteins may be influenced as well. Construction of protein networks based on protein expression levels should consider variations of protein expression in cluster proteins in postmortem brain. Probably, preservation or degradation may be very different from one protein to another thus hampering any conclusion when one protein is elevated and the associated protein is reduced within a context in which the reduced protein is very vulnerable to PMI.

Additional aim of our study to investigate epigenetic markers alterations with prolonged PMI and storage duration as well as to TRP level, revealed that methyltransferase and acetyltransferase activities were relatively preserved with PMI and storage duration. The silencing of epigenetically regulated promoters is associated with high levels of DNA methylation and decreased acetylation of core histones in their regions (Li and Bird 2007). Such epigenetic down-regulation of genes are investigated in mechanisms involved in the GABAergic neurons in schizophrenia (Kundakovic et al. 2009), as well as in Tauopathies including Alzheimer's disease (Barrachina and Ferrer 2009). Here, we could show that there are probably no limitation using human postmortem brain tissue for epigenetic studies. Still it would be important to confirm these results in an independent laboratory with independent brain tissue samples, as well as to investigate whether in artificial PMI conditions with time and different storage conditions these two enzyme stay stable. In addition, validation in epigenetic brain research would be of interest for further confirmation of effects through pre-mortem and postmortem conditions.

In conclusion, in this study we could demonstrate that for PMI and storage duration one may use TRP levels as a quality control marker, since some proteins seems to be sensitive to prolonged PMI and storage duration. Via elevated TRP levels the general degradation of proteins might be evaluated, before evaluation of the specific protein/enzyme of interest. Therefore, scientists using human postmortem brain tissue for proteomic studies should take in account such markers. On the other hand, studying epigenetic alterations seems to be independent of PMI and storage duration, offering scientists a broad usage of postmortem brain tissue for their investigations.

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